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Serpentine cAMP Receptors May Act through a G Protein–Independent Pathway to Induce Postaggregative Development in Dictyostelium

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Summary

The transcription factor G box–binding factor (GBF) is required for the developmental switch between aggregative and postaggregative gene expression, cell-type differentiation, and morphogenesis. We show that constitutive expression of GBF allows ectopic expression of postaggregative genes, but only in response to exogenous cAMP. GBF activation requires the serpentine cAMP receptors required for aggregation, but not the coupled Gα2 or the Gβ subunit, suggesting a novel signaling pathway. In response to high cAMP, *gα2*-null cells can bypass the aggregation stage, expressing cell type–specific genes and forming fruiting bodies. Our results demonstrate that the same receptors regulate aggregation and cell-type differentiation, but via distinct pathways depending upon whether the receptor perceives a pulsatile or sustained signal.

Introduction

Multicellular development in Dictyostelium discoideum can be loosely separated into two phases. In the first, undifferentiated cells chemotax toward nanomolar pulses of cAMP to form a multicellular aggregate. In the second phase, cells within the aggregate are induced to differentiate into prestalk and prespore cell populations, expressing cell type–specific genes (which function as molecular markers). This process leads to morphogenesis, transforming the aggregate into a migrating pseudoplasmodium and, ultimately, a fruiting body. The aggregation-stage responses, including the activation of adenylyl and guanylyl cyclases, which control signal relay, chemotaxis, and early, pulse-induced gene expression, are mediated by nanomolar pulses of cAMP acting through the cell surface, serpentine cAMP receptor 1 (cAR1), and the coupled G protein containing the Gα subunit Gα2 (Devreotes, 1994). When the aggregate forms, levels of cAMP rise from nanomolar to micromolar levels (Abe and Yanagisawa, 1983), and these high, more continuous levels of cAMP repress expression of the aggregation-stage genes and induce expression of the postaggregative genes (Mann and Firtel, 1987; Mehdy and Firtel, 1985; Schaap and van Driel, 1985; Town and Gross, 1978). Postaggregative genes, the first to be expressed during the second phase of development, can be directly activated by cAMP in suspension assays in the absence of cell–cell contacts, whereas cell type–specific genes (prestalk- and prespore-specific genes) require cAMP as well as additional signals afforded by cell–cell contacts, postaggregative gene products (in the case of prestalk genes), the morphogen DIF (Berks and Kay, 1990; Dynes et al., 1994; Jermyn et al., 1987; Mehdy and Firtel, 1985; Williams et al., 1987), or some combination of these.

The G box–binding factor (GBF) is a cAMP-induced DNA-binding activity that binds to *cis* elements required for the developmental and cAMP-mediated induction of postaggregative and cell type–specific genes and whose expression is also induced by cAMP (Schnitzler et al., 1994). These *cis* elements were initially identified in prestalk-enriched gene promoters, but similar cAMP response elements from prespore promoters and a prestalk-specific gene have also been shown to function as GBF-binding sites (Ceccarelli et al., 1992; Datta and Firtel, 1988; Fosnaugh and Loomis, 1993; Haberstroh and Firtel, 1990; Hjorth et al., 1989, 1990; Pears and Williams, 1988; Powell-Coffman et al., 1994; Schnitzler et al., 1994). In studies using wild-type and mutated elements, in natural promoter contexts, in heterologous promoters, or in some combination of these, there is a direct correlation between the ability of the *cis* elements to direct developmental and cAMP-induced expression in vivo and its affinity for GBF in vitro (Ceccarelli et al., 1992; Hjorth et al., 1989, 1990). GBF-binding sites are necessary but not sufficient for cAMP or developmental induction of postaggregative genes. GBF is not cell type specific, and additional promoter elements are necessary to allow GBF-binding sites to confer appropriate temporal and cell type–specific patterns of expression (Ceccarelli et al., 1992; Hjorth et al., 1990; Powell-Coffman et al., 1994).

The *GBF* gene has recently been cloned and encodes a novel, putative zinc finger protein. Cells in which the *GBF* gene has been disrupted (*gbf*-null cells) aggregate normally but arrest in their development at the loose aggregate stage (Schnitzler et al., 1994). The aggregates synchronously disperse and then reaggregate in cycles, but never proceed past the loose aggregate stage. The initial induction of early, pulse-induced genes during aggregation, and their subsequent repression upon formation of the mound is normal. As the mound disperses and then reaggregates, these genes are reinduced and then rerepressed. In contrast, *gbf*-null cells do not induce the expression of postaggregative or cell type–specific genes, either when plated for development or in suspension culture in response to cAMP. Expression of GBF from the strong early promoter actin 15 (*Act15*) complements the inability of the null cells to express postaggregative genes and results in high levels of GBF protein in vegetative amoebae.

Previous pharmacological analysis has indicated that postaggregative gene expression is induced by extracellular cAMP through cell surface receptors (Gomer et al.,

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1985; Haribabu and Dottin, 1986; Oyama and Blumberg, 1986; Schaap and van Driel, 1985). In this paper, we show that constitutive expression of GBF is not sufficient to induce postaggregative gene expression, but that this requires a potentially novel signaling pathway that is activated through the same cARs that control aggregation but does not require the Gα subunit or the Gβ subunit that is required for other aspects of development. We further demonstrate that cells lacking the Gα2 subunit (*gα2*-null cells), which cannot aggregate and show no activation of adenylyl or guanylyl cyclases, can be induced to express the entire set of postaggregative and cell type–specific genes and to differentiate into fruiting bodies if treated with cAMP and plated on a substratum. Our data indicate that distinct receptor-mediated pathways differentially regulate early and postaggregative development in Dictyostelium. Furthermore, our results indicate that presentation of a ligand in the form of a high, sustained versus low, pulsatile signal can induce different signaling pathways within the same cell, with distinct, downstream biological consequences. This highlights a potentially novel mechanism by which receptors can control cell function.

Results

A cAMP Signaling Pathway and GBF Are Necessary to Induce Postaggregative Gene Expression

GBF is expressed at very low levels in vegetatively growing wild-type cells and is rapidly induced during development at the time of mound formation or in suspension culture by high cAMP, conditions in which postaggregative gene expression is induced (see Introduction). We therefore examined whether constitutive expression of GBF in vegetative cells is sufficient to induce postaggregative gene expression. As shown in Figure 1, the expression of two such genes, *LagC* and *CP2*, was not detected in wild-type vegetative cells (lane 1) and was only barely detectable in *gbf*-null vegetative cells constitutively expressing GBF (lane 5), indicating that increasing GBF levels is not sufficient to induce these postaggregative genes. However, when cAMP was added for 90 min to wild-type cells constitutively expressing GBF (Figure 1, lane 4) or to *gbf*-null cells constitutively expressing GBF (lane 6), both *LagC* and *CP2* were rapidly induced (see below). Similar results were observed with a third postaggregative gene, *rasD* (data not shown). In contrast, control (untransformed) wild-type cells do not induce the expression of these genes under the same conditions (Figure 1, lane 2); they require a period of starvation of 6 hr followed by exposure to cAMP for 3–6 hr (Mehdy and Firtel, 1985) (see below). These results demonstrate that postaggregative gene induction requires cAMP-mediated activation of a signaling pathway in addition to GBF, presumably to activate GBF, and that vegetative cells constitutively expressing GBF have all of the components of this signal transduction pathway.

Figure 2 shows the kinetics of induction of the postaggregative gene *LagC* in *gbf*-null cells constitutively expressing GBF in response to cAMP. *LagC* expression can

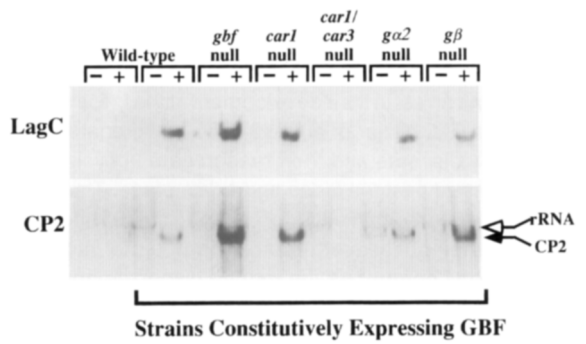

Figure 1. Induction of the Postaggregative Genes *CP2* and *LagC* in Response to cAMP

Wild-type cells (strain KAx-3) and mutant strains were transformed with the *Act15–GBF* vector, and stable transformants were isolated. Vegetatively growing cells of these strains and control (untransformed) wild-type cells were washed and placed in nonnutrient buffer in suspension and shaken in the presence (plus) or absence (minus) of 300 μM cAMP for 90 min (Mehdy and Firtel, 1985). RNA was isolated and analyzed by RNA blot hybridization (Schnitzler et al., 1994). The *gbf*-null strain not constitutively expressing GBF shows no induction of postaggregative genes under any conditions (Schnitzler et al., 1994).

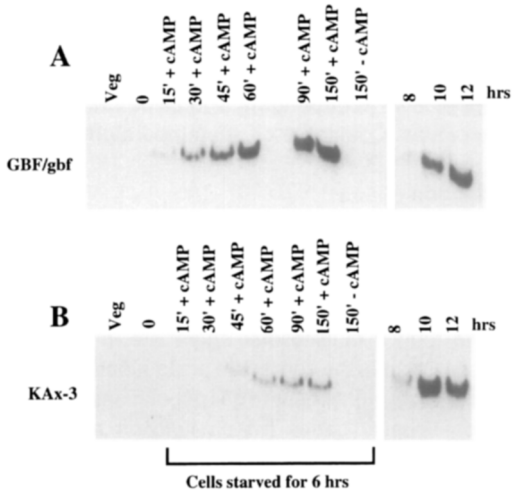

Figure 2. Kinetics of Induction of *LagC*

(A) *gbf*-null cells constitutively expressing GBF were placed in suspension culture under conditions that minimize cell–cell contacts (Dynes et al., 1994) and assayed for *LagC* expression in the presence and absence of cAMP as described in the legend to Figure 1. Cells were washed and placed in suspension culture. cAMP was added to the cultures at 30 min. This sample is labeled 0. Samples were then taken at the times indicated. Veg indicates vegetatively growing cells and is a sample taken prior to placing the cells into suspension. RNAs from complimented *gbf*-null cells plated for development and taken between the postaggregative aggregate/early mound stage (8 hr) and tipped aggregate stages (12 hr) are shown as markers for levels of expression during normal development.

(B) Wild-type cells were placed in suspension and shaken for 6 hr as described for (A) prior to the addition of cAMP and then assayed for *LagC* gene expression. RNAs from wild-type cells plated for development and taken between the postaggregative aggregate/early mound stage (8 hr) and tipped aggregate stages (12 hr) are shown as markers for levels of expression during normal development. All lanes contained the same amount of total cell RNA. The filters were hybridized with the same probe so that the absolute level of expression can be compared among all the samples.

be seen as early as 15 min after addition of cAMP and reaches a maximum by 60 min, after which the level remains constant over the 2.5 hr timeframe of the experiment. This level of expression is similar to that seen at the tipped aggregate stage (12 hr of development) when *gbf*-null cells constitutively expressing GBF or wild-type cells are plated on filters for multicellular development. Under these experimental conditions, *LagC* expression can first be detected at 8 hr of development, the time of initial mound formation in these experiments. When wild-type cells are starved in suspension for 6 hr without exogenous cAMP and then induced with cAMP under conditions that minimize cell–cell contacts (Mehdy and Firtel, 1985), *LagC* gene expression is not observed until 45 min, and the level of expression is lower than that seen in the cells constitutively expressing GBF. Maximal expression is observed after 4–6 hr of cAMP stimulation (Dynes et al., 1994). No induction is seen at 2.5 hr if the cells are shaken in the absence of cAMP. If cAMP is given prior to 4 hr of starvation, no induction is seen (data not shown).

cAMP/GBF Induction of Postaggregative Genes Is Receptor-Dependent and Independent of Known G Protein Subunits

The cloning of the gene encoding GBF offers an opportunity to trace the cAMP signal cascade from the cell surface to transcriptional activation in Dictyostelium. Genes encoding a number of signal transduction components required for multicellular development have been cloned, and null mutants have been created by homologous recombination. Many of these strains are deficient in postaggregative gene expression, and development is blocked at or before the mound stage, the stage at which *gbf*-null cells arrest, suggesting a potential role of these genes in GBF-mediated, cAMP-induced activation of postaggregative gene expression. The failure of these mutants to express postaggregative genes properly could be due to a number of factors: the cells may fail to aggregate, preventing the increase in cAMP levels required for postaggregative gene expression, they may fail to express GBF properly, or they may be deficient in a component of the signal transduction pathway (other than GBF) required for cAMP-induced postaggregative gene expression. By assaying gene expression in vegetative cells constitutively expressing GBF with and without exposure to exogenous cAMP, we can potentially eliminate the first two factors, bypassing the normal developmental requirements for postaggregative gene expression, and focus on the third to determine directly which specific signaling components are required for the cAMP-mediated activation of GBF function. Such an assay investigates fairly rapid responses, thereby minimizing the likelihood of secondary effects due to newly induced gene expression.

The *Act15–GBF* expression vector was stably transformed into strains carrying null mutations of known signal transduction components, and the cAMP-induced expression of *LagC*, *CP2*, and *rasD* was examined using the 90 min suspension assay. cAR1 is the principal cAR responsible for cAMP pulse relay, chemotaxis, and pulse-induced gene expression during aggregation, which is induced by 30 nM levels of cAMP (Sun and Devreotes, 1991). Aggregation-stage cells also express a second, lower-affinity cAR, cAR3. Recent studies have shown that, while *car1*-null cells do not aggregate under normal conditions, they can develop if initially given higher (300 nM) pulses of cAMP followed by continuous higher levels of cAMP and then plated (Insall et al., 1994; Soede et al., 1994). Figure 1 shows that cAMP successfully induces postaggregative gene expression in vegetative *car1*-null cells constitutively expressing GBF. However, the *car1/car3* double knockout strain is nonresponsive to extracellular cAMP, indicating that the response is receptor dependent. Because *car3*-null cells have been previously shown to express wild-type levels of postaggregative and cell type–specific genes and to develop normally (Johnson et al., 1993) (presumably the function of cAR3 is redundant), they were not tested. Thus, these results indicate that cAMP-mediated postaggregative gene induction requires cARs and that either cAR1 or cAR3 can function in this capacity.

We then investigated the participation of G proteins in this pathway. In almost all cases, G proteins mediate the signal transduction downstream from serpentine receptors, such as cAR1 and cAR3, to downstream effectors (see Discussion). Genes encoding eight Dictyostelium Gα subunits have been cloned, and seven (all but Gα6) have been disrupted by homologous recombination (Devreotes, 1994; Dharmawardhane et al., 1994; Hadwiger and Firtel, 1992; Wu et al., 1994a, 1994b). Of these, only *gα2*-null cells arrest prior to the tipped aggregate stage. These *gα2*-null cells lack cAMP-mediated activation of adenylyl and guanylyl cyclases and phospholipase C, and they do not express postaggregative genes when plated for development (Kesbeke et al., 1988; Kumagai et al., 1989, 1991). *Act15–GBF/gα2*-null cells, however, express *CP2* and *LagC* in response to cAMP in suspension culture after 45 or 90 min (see Figure 1; data for 45 min not shown). This finding does not rule out a requirement for heterotrimeric G proteins, as one of the many other Gα subunits may substitute for Gα2. However, recent results suggest that D. discoideum has only a single G protein β subunit (Lilly et al., 1993; Wu et al., 1995; see Discussion). *gβ*-null cells do not aggregate and lack all previously examined cAR–mediated pathways except Ca^{2+} influx (Lilly et al., 1993; Milne et al., 1995; Wu et al., 1995; see Discussion). *gβ*-null cells constitutively expressing GBF show activation of the postaggregative genes in response to cAMP (see Figure 1). Similar results were obtained for *rasD* (data not shown). These results indicate that the cAMP induction of postaggregative genes does not require the Gα or Gβ subunit that is required for aggregation (see Discussion).

Extracts were made from all of the above cell lines, and levels of GBF DNA-binding activity were determined by gel shift analysis (Schnitzler et al., 1994; Figure 3). In each cell line carrying the *Act15–GBF* expression plasmid, the level of GBF-binding activity was 9- to 30-fold higher than that found in vegetative wild-type cells, although lower than that found in wild-type cells at the mound stage, when in vivo levels are maximal (data not shown).

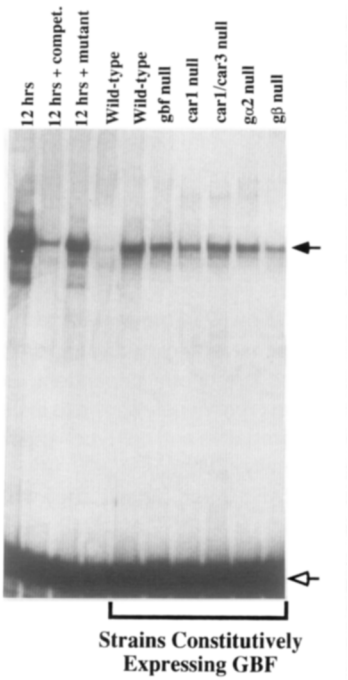

Figure 3. Gel Shift Analysis of GBF-Binding Activity in Strains Used in Figure 1

Extracts and binding activity were analyzed as previously described (Schnitzler et al., 1994). As a control, extracts from wild-type cells developed for 12 hr (lane 1) are shown in the presence of specific competitor (lane 2) and mutant competitor that binds GBF poorly (lane 3) (Schnitzler et al., 1994) to show relative mobility of the GBF-specific band. Closed arrowhead, specific binding activity; open arrowhead, uncomplexed probe.

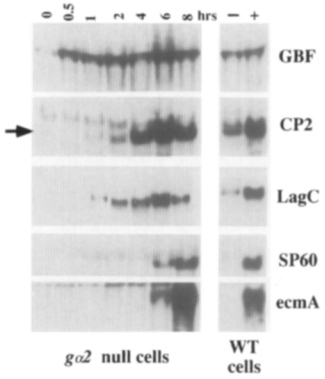

Figure 4. Kinetics of Postaggregative and Cell Type–Specific Gene Expression in *gα2*-Null Cells in Suspension Culture

Expression was assayed in suspension culture, which allows the role of extracellular cAMP and other morphogens to be assayed (Mehdy and Firtel, 1985). *gα2*-null cells were shaken in suspension culture at 125 rpm for 6 hr in morpholineethanesulfonic acid (MES)–pad dilution fluid (PDF) nonnutient buffer (Mehdy and Firtel, 1985). Under these conditions, cell–cell contacts form. cAMP was then added to 300 μM and supplemented with 100 μM cAMP every 2 hr. RNA was isolated at the timepoints indicated, separated on denaturing gels, blotted, and probed with the DNAs indicated. The 0 timepoint is the time of cAMP addition (6 hr after starvation). As a control, wild-type (WT) cells were also starved for 6 hr and then shaken for 6 hr in the presence (plus) and absence (minus) of cAMP. Under these conditions, GBF and the postaggregative genes *CP2* and *LagC* are induced in the absence of exogenous cAMP owing to production of cAMP in the cell agglomerates that form. The cell type–specific genes *ecmA* (prestalk) and *SP60* (prespore) require additional signals as well as exogenous cAMP (Berks and Kay, 1990; Dynes et al., 1994; Mehdy and Firtel, 1985). *gα2*-null cells show no induction in the absence of added cAMP (data not shown) since they are unable to activate adenylyl cyclase and produce endogenous cAMP.

Gα2 Is Not Essential for Multicellular Development

Our observation that postaggregative gene expression is induced by cAMP in vegetative *Act15–GBF/gα2*-null cells contrasts with earlier results showing the absence of postaggregative gene expression in *gα2*-null cells plated on filters for development, conditions in which there is no exogenous cAMP (Kumagai et al., 1991). This led us to assay *gα2*-null cells (not carrying *Act15–GBF*) in suspension for postaggregative and cell type–specific gene expression. We found that *GBF*, the postaggregative genes *CP2* and *LagC*, and the cell type–specific genes *ecmA* (prestalk) and *SP60* (prespore) are expressed at wild-type levels in suspension culture in response to exogenous cAMP (Figure 4). Moreover, the kinetics of induction of these genes are consistent with that observed in wild-type cells during multicellular development (Dynes et al., 1994; Mehdy et al., 1983; Pears and Williams, 1987; Schnitzler et al., 1994). Following 6 hr of starvation, *GBF* transcripts are induced most rapidly in response to cAMP addition, reaching maximal levels within 30 min, consistent with it being essential for the expression of the other genes. Expression of *LagC* and *CP2* is first detected within 1 hr, while the cell type–specific gene transcripts are initially detected at 4 hr, in accord with the requirement of *LagC* or other postaggregative gene products for their expression (Dynes et al., 1994). No expression is observed in the absence of cAMP (data not shown). If cAMP is added at the outset, rather than following 6 hr of starvation, the expression of the cell type–specific genes is not detected until 6 hr, indicating a period of starvation is required. During this time, *GBF* transcripts and DNA-binding activity increase severalfold (Schnitzler et al., 1994; data not shown).

When *gα2*-null cells (not constitutively expressing GBF) were starved, shaken with cAMP, and subsequently plated for development, cells "coalesced" into small aggregates that then formed small, fairly normal-looking fruiting bodies with viable *gα2*-null spores (Figure 5). Recently, *car1*-null cells have also been shown to induce postaggregative genes in suspension culture and subsequently to form fruiting bodies, presumably by employing other cARs, such as cAR3 (Soede et al., 1994). Together, these results indicate that the signal transduction molecules required for aggregation are largely separate from those required for postaggregative and cell type–specific gene induction. Furthermore, they indicate that many aggregation-stage genes are dispensible if, other than aggregation, another route to postaggregative and cell type–specific gene induction is provided. By contrast, when we tested *gβ*-null cells in suspension cultures using the same conditions, no postaggregative genes were expressed and cells did not form aggregates (data not shown). In *gβ*-null cells con-

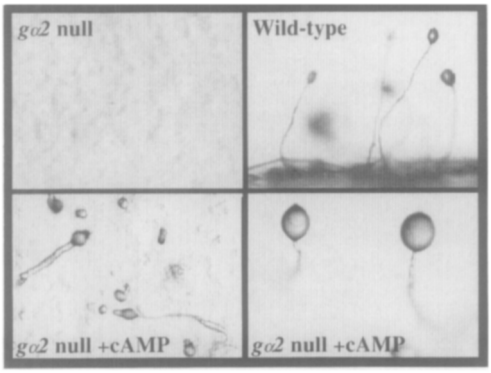

Figure 5. Photographs of Wild-Type and *gα2*-Null Cells Plated for Development under Different Conditions

The upper left panel shows *gα2*-null cells that were washed and plated for development. No aggregation is seen as previously described (Kumagai et al., 1991). The upper right panel shows wild-type fruiting bodies at 26 hr. The lower panels show *gα2*-null cells that were shaken for 6 hr, given cAMP for 6 hr (as described in the legend to Figure 3), and then plated for development. The magnification shown in the panel at the lower right is ~5× that of the upper right panel. The lower right panel shows an enlargement of the sori. These contain mature spores.

stitutively expressing GBF, which induced postaggregative genes in response to cAMP, the cell type–specific genes were not induced (see Figure 1; data not shown; see Discussion).

To examine the separate requirements for early and postaggregative gene expression further, we examined the cAMP pulse-induced expression of *D2* in wild-type and *gbf*-null cells. In wild-type cells, early gene expression is induced in response to 30 nM pulses of cAMP and requires cAR1 (Mann and Firtel, 1987; Soede et al., 1994) but can be activated in *car1*-null cells in response to 300 nM pulses, owing to signal transduction through cAR3, which can only respond to higher levels of cAMP (Johnson et al., 1992). In wild-type and *car1*-null strains, *D2* expression is repressed by high, continuous levels (300 μM) of cAMP (Mann and Firtel, 1987, 1989). We tested whether the repression of *D2* by high, continuous levels of cAMP is dependent on either GBF or the induction of GBF-dependent genes. Figure 6 shows that *D2* is induced to very high levels by 30 nM pulses of cAMP in *gbf*-null cells and that these cells have the characteristic repression of *D2* by a continuous (nonpulsatile) 300 μM cAMP signal that activates postaggregative genes and down-regulates early pathways (Devreotes, 1994; Mann and Firtel, 1987; Mehdy and Firtel, 1985; Schaap and van Driel, 1985; Soede et al., 1994). The magnitude of its repression is the same in *gbf*-null cells as that described previously for wild-type cells (Mann and Firtel, 1989). This is consistent with our observations that pulse-induced gene expression is normally repressed upon mound formation in *gbf*-null strains (Schnitzler et al., 1994).

Discussion

At the mound stage, Dictyostelium cells repress aggregation-stage gene expression and activate a developmental

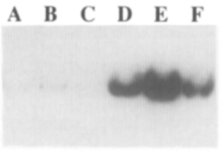

Figure 6. Inhibition of Pulse-Induced Gene Expression in *gbf*-Null Cells

Vegetative cells were washed and shaken in suspension in nonnutrient buffer (MES–PDF) (Mann and Firtel, 1987). Cells were either shaken without exogenous cAMP, with 30 nM cAMP pulses given every 6 min, or with 300 μM cAMP (supplemented every 2 hr). RNA was isolated at the times indicated, sized on denaturing gels, and probed with the pulse-induced gene *D2* (Mann and Firtel, 1987). Lane A, RNA from vegetative cels; lane B, RNA from cells shaken without cAMP and harvested at 6.5 hr; lane C, RNA from cells shaken with 300 μM cAMP and harvested at 6.5 hr; lane D, RNA from cells shaken and pulsed with 30 nM cAMP and harvested at 4.5 hr; lane E, RNA from cells shaken and pulsed with 30 nM cAMP and harvested at 6.5 hr; lane F, cells shaken and pulsed with 30 nM cAMP for 4.5 hr. cAMP was added to 300 μM. RNA was isolated from cells harvested at 6.5 hr.

cascade that results in cell-type differentiation. Prior to this, pulses of cAMP direct the chemotaxis of cells to form the aggregate. As the aggregate forms, the rising levels of cAMP within the mound result in the GBF-independent inhibition of the signaling pathways that led to aggregation, including repression of pulse-induced gene expression, and in the GBF-dependent induction of the postaggregative genes. *LagC* and possibly other postaggregative genes are then required for expression of the prestalk- and prespore-specific genes and for cell-type differentiation (Dynes et al., 1994). GBF itself, present at low levels in starved cells, is rapidly induced at the time of initial mound formation, in what we have proposed to be a GBF-dependent autoregulatory loop (Hjorth et al., 1989; Schnitzler et al., 1994). Our results suggest that the signal transduction apparatus required for postaggregative gene expression is already present in wild-type cells and that GBF and cAMP levels act jointly as a timing mechanism. As cAMP levels rise, GBF expression increases, with positive feedback playing a role in its induction. The high levels of GBF can then respond to cAMP-initiated signal transduction to induce expression of the postaggregative genes. Moreover, in *gbf*-null cells, repression of the pulse-induced aggregation-stage genes occurs normally with the formation of the mound (Schnitzler et al., 1994), indicating it may be the rise of cAMP levels in the mound (Abe and Yanagisawa, 1983) and not postaggregative gene expression that represses the expression of the pulse-induced aggregation-stage genes.

We have observed that the induction of the postaggregative gene *LagC* occurs very rapidly in cells constitutively expressing GBF and can rapidly reach the same level of expression observed during multicellular development. This observation would suggest that this induction is an immediate response to the signal transduction pathway and does not require intermediate steps. One direct test for this is to examine whether the process occurs in the presence of protein synthesis inhibitors. Experiments indicate that addition of cycloheximide 30 min before the addition of cAMP results in no significant accumulation of *LagC*

or *CP2* transcripts after 90 min (G. R. S. et al., unpublished data). While it remains possible that an intermediate gene product induced by cAMP in cells constitutively expressing GBF is required for postaggregative gene expression, this is not consistent with the very rapid induction of *LagC*. We expect that one or more of the gene products required for the GBF response are either rapidly turned over or that the *CP2* and *LagC* transcripts are unusually unstable when protein synthesis is blocked at this stage of development.

Data presented here and previous observations (see Introduction) demonstrate that a cell surface seven-transmembrane domain cAR (either cAR1 or cAR3) is required to activate the GBF-mediated expression of postaggregative genes, as well as aggregation-stage responses. The fact that we see only a small decrease in the level of postaggregative gene expression in *car1*-null cells (which express cAR3) relative to wild-type cells suggests that cARs are not limiting and that relatively few receptors are required to activate this pathway; that of cAR3, though present in lower numbers, is more efficient in activating this response than cAR1. We furthermore show that neither the critical early developmental Gα subunit Gα2 nor the only known Dictyostelium Gβ subunit (see below) is required for the signal transduction pathway leading to postaggregative gene expression. The lack of a requirement for Gα2 is consistent with the observation that a Gα2 dominant negative subunit, which completely blocks aggregation, has no detectable effect on cell type–specific gene expression (Carrel et al., 1994). Although it is possible that some of the Gα subunits have redundant functions, the fact that they share no more homology with each other than with Gα subunits from other systems argues against this. Moreover, disruption of the genes encoding six of the other seven known Gα proteins does not affect development prior to the tipped aggregate stage (Devreotes, 1994; Dharmawardhane et al., 1994; Hadwiger and Firtel, 1992; Kumagai et al., 1991; Wu et al., 1994b). (The gene encoding Gα6 has not been disrupted, but its expression is restricted to growth and very early development, and it is not detectably expressed in the multicellular stages [Cubitt et al., 1992; Wu and Devreotes, 1991].) Interestingly, GBF-mediated postaggregative gene expression is not activated by folate (G. R. S. et al., unpublished data), which activates guanylyl cyclase and chemotaxis through a distinct non-cAR that couples to the Gα subunit Gα4 (Hadwiger et al., 1994).

Significant evidence exists that Dictyostelium has only a single G protein β subunit (Lilly et al., 1993). *gβ*-null cells lack all known receptor-mediated/G protein–dependent pathways, including cAMP or postaggregative activation of guanylyl cyclase and chemotaxis (Wu et al., 1995). The lack of stimulation by either chemoattractant is significant since they are mediated by two distinct Gα subunits, Gα2 and Gα4 (Hadwiger et al., 1994; Kesbeke et al., 1988; Kumagai et al., 1991). Moreover, as shown by Scatchard analysis, membranes from *gβ*-null cells have only low affinity cAMP-binding sites that are unaffected by GTP, and *gβ*-null cells lack cAMP/GTPγS activation of adenylyl cyclase in vitro. However, *gβ*-null cells expressing GBF strongly induce the postaggregative genes following starvation and treatment with cAMP. These results suggest a model in which cAR/GBF-mediated postaggregative gene expression functions through a pathway that may be G protein independent. In our analysis, however, we cannot exclude the presence of another Gβ protein subunit that is not detected in the above assays because it is expressed at very low levels and thus does not significantly contribute to the number of high affinity sites or the GTPγS effect on cAMP binding or because it has unusual biochemical properties. Our results, however, do clearly indicate that the pathways and G proteins that are required for the responses during aggregation and for those mediating postaggregative gene expression are distinct.

We are aware of only two instances of putative G protein–independent, serpentine receptor–mediated signaling that have been defined genetically. The first is a yeast pheromone-induced response (Jackson et al., 1991), and the second is the receptor-mediated calcium influx in Dictyostelium (Milne et al., 1995). Furthermore, the mammalian angiotensin II receptor does not show the classic change in agonist affinity in the presence of guanine nucleotides, suggesting that it may not couple to G proteins (Mukoyama et al., 1993). This receptor is differentially regulated during mouse development and has been proposed to be involved in developmental decisions (Mukoyama et al., 1993). Our data suggest that the induction of postaggregative gene expression is another example of putative G protein–independent signaling by a seven-span receptor. In Dictyostelium, calcium ionophores and channel blockers have previously been shown to affect the expression of some postaggregative and cell type–specific genes (Blumberg et al., 1988, 1989; Kumagai et al., 1988; Schaap et al., 1986), and an increase in cytosolic calcium has been observed in aggregates in the mound at the time of cell type–specific gene induction (Kubohara and Okamoto, 1994; Saran et al., 1994; Schlatterer et al., 1992). These data suggest a potential role for calcium influx in GBF-mediated postaggregative gene regulation.

Biochemical studies have shown that in mammalian cells, activation of rhodopsin or β-adrenergic receptors is sufficient to allow the respective receptor kinases to bind to and phosphorylate the receptor, in a G protein–independent process, while unstimulated receptors do not bind the kinase (Chen et al., 1993; Palczewski et al., 1991). Our data suggest a pathway in which the continuous binding of the ligand to cARs recruits cytosolic components to the plasma membrane, where they bind to the receptor and organize a signaling cascade in a manner analogous to the recruitment of signaling components to the plasma membrane in receptor tyrosine kinase–mediated pathways.

The level of expression of the postaggregative genes in the *gβ*- and *gα2*-null strains is somewhat lower than we observe for wild-type cells expressing *Act15–GBF*, suggesting that a second signal transduction pathway involving heterotrimeric G proteins may be needed for maximal expression, but is not essential. There are also differences in the relative level of expression of two postaggregative genes (*CP2* and *LagC*) in some strains, suggesting that other pathways may provide input into the expression of individual genes.

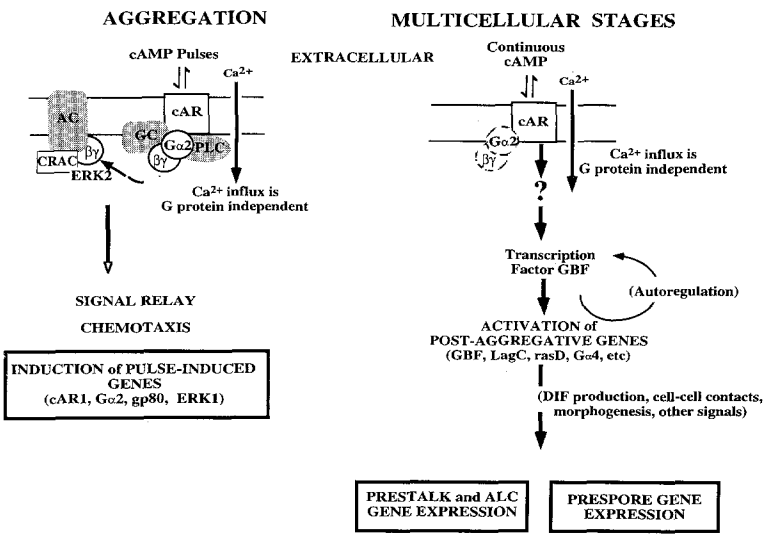

Figure 7. Models for cAR-Mediated Pathways during Aggregation and Multicellular Development

During aggregation, cAMP binds to cAR1 and activates guanylyl cyclase (GC), phospholipase C (PLC), adenylyl cyclase (AC), and pulse-induced gene expression (see Introduction). In vivo, all of these processes require the G protein containing the Gα2 subunit and Gβ. The pathway is drawn with a direct coupling of guanylyl cyclase and phospholipase C to the G protein containing Gα2, although this has not been proven. Adenylyl cyclase is thought to be activated by Gβγ, and this activation is known to require CRAC, the cytosolic activator of adenylyl cyclase, and the MAP kinase ERK2 (Lilly et al., 1993; Lilly and Devreotes, 1994; Segall et al., 1995). In addition, cAMP binding results in a Ca^{2+} influx, which is G protein independent (Milne et al., 1995). A role for this influx has not been shown. In the multicellular stages, activation of postaggregative gene expression occurs through cARs but is either independent of G proteins or is mediated through a distinct set of G proteins than those that regulate aggregation. cAMP response pathways similar to those present during aggregation also occur and may regulate morphogenesis during tip formation and culmination through Gα2. A low level of GBF is expressed during starvation. As the mound forms, cAMP levels increase. This increase activates GBF and downstream gene expression (postaggregative genes) through a cAR-mediated signaling pathway that may involve the posttranslational modification of GBF or an associated protein. This induces the postaggregative genes, including the putative cell surface protein LagC, which is required for prestalk/prespore expression (Dynes et al., 1994). DIF is required for the prestalk pathway and inhibits the prespore pathway (Kay, 1992; Williams, 1991).

We show that Gα2 and the known Gβ, which are absolutely required for aggregation-stage responses, are not essential for the cAMP induction of postaggregative genes in cells overexpressing GBF. Furthermore, we demonstrate that *gα2*-null cells, when provided exogenous cAMP, activate the expression of postaggregative and cell type–specific genes, including *GBF*, and form fruiting bodies in a near-normal fashion. Pulse-induced gene regulation and other aggregation-stage responses are nearly wild type in *gbf*-null cells, although GBF is absolutely required for postaggregative gene expression. Together, these results suggest that two distinct receptor-mediated signal transduction pathways, one functioning through Gα2 and the other through a possible G protein–independent mechanism, regulate distinct stages of Dictyostelium development. A model for the receptor-mediated aggregation-stage and postaggregation-stage signaling pathways is presented in Figure 7. Interestingly, *gβ*-null cells can activate postaggregative gene expression in response to cAMP, but do not induce the cell type–specific genes, suggesting that a G protein–dependent (but not requiring Gα2) pathway is required for later development. This is consistent with an earlier finding that the Gα4 subunit (which is induced in the mound stage and couples to postaggregative receptors) is required for high levels of prestalk and prespore gene expression (Hadwiger and Firtel, 1992). Since *gβ*-null cells would be defective in all Gα subunit–requiring pathways, we expect that a combination of pathways regulated by G proteins is necessary for cell type–specific gene expression and morphogenesis. In addition, the *gβ*-null cells have a growth defect (Wu et al., 1995), and it is possible that the inability of these cells to respond fully may be related to other pleiotrophic effects of the mutation.

By having two distinct receptor-mediated pathways, the same receptor and ligand can be used throughout aggregation and morphogenesis, with the formation of the mound and increasing cAMP levels acting as a developmental cue to induce cell-type differentiation. cAR1, cAR3, or both probably respond to these high concentrations of cAMP in the mound, coupling to a largely distinct signaling cascade to activate GBF to induce the postaggregative genes. These genes then direct cellular differentiation and postaggregation morphogenesis. The aggregation-stage receptor-mediated pathway is specialized to respond to nanomolar-level pulsatile signals, allowing aggregation-stage responses and gene expression, whereas the postaggregative pathway is specialized to respond to high, continuous cAMP levels to induce gene expression through GBF. The ability of vegetative cells to induce postaggregative gene expression when GBF is constitutively expressed indicates that the signaling componenets for this process are already present in cells at the earliest stages of development.

Experimental Procedures

Cell Culture and Molecular Biological Procedures

All cell culture procedures and molecular techniques used have been described previously. Individual details are referenced in the figure legends.

Construction of Strains Constitutively Expressing GBF

The *Act15–GBF* expression vector has been described previously (Schnitzler et al., 1994). Individual strains were transformed with this vector, selecting for G418 resistance (Schnitzler et al., 1994).

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References

Abe, K., and Yanagisawa, K. (1983). A new class of rapid developing mutants in *Dictyostelium discoideum*: implications for cyclic AMP metabolism and cell differentiation. Dev. Biol. *95*, 200–210.

Berks, M., and Kay, R. R. (1990). Combinatorial control of cell differentiation by cAMP and DIF-1 during development of *Dictyostelium discoideum*. Development *110*, 977–984.

Blumberg, D. D., Comer, J. F., and Higinbotham, K. G. (1988). A Ca^{++}-dependent signal transduction system participates in coupling expression of some cAMP-dependent prespore genes to the cell surface receptor. Dev. Genet. *9*, 359–370.

Blumberg, D. D., Comer, J. F., and Walton, E. M. (1989). Ca^{++} antagonists distinguish different requirements for cAMP mediated gene expression in the *Dictyostelium discoideum*. Differentiation *41*, 14–21.

Carrel, F., Dharmawardhane, S., Clark, A. M., Powell-Coffman, J. A., and Firtel, R. A. (1994). Spatial and temporal expression of the *Dictyostelium discoideum* Gα protein subunit Gα2: expression of a dominant negative protein inhibits proper prestalk to stalk differentiation. Mol. Biol. Cell *5*, 7–16.

Ceccarelli, A., Mahbubani, H. J., Insall, R., Schnitzler, G., Firtel, R. A., and Williams, J. G. (1992). A G-rich sequence element common to *Dictyostelium* genes which differ radically in their patterns of expression. Dev. Biol. *152*, 188–193.

Chen, C.-Y., Dion, S. B., Kim, C. M., and Benovic, J. L. (1993). β-Adrenergic receptor kinase: agonist-dependent receptor binding promotes kinase activation. J. Biol. Chem. *268*, 7825–7831.

Cubitt, A. B., Carrel, F., Dharmawardhane, S., Gaskins, C., Hadwiger, J., Howard, P., Mann, S. K. O., Okaichi, K., Zhou, K., and Firtel, R. A. (1992). Molecular genetic analysis of signal transduction pathways controlling multicellular development in *Dictyostelium*. Cold Spring Harbor Symp. Quant. Biol. *57*, 177–192.

Datta, S., and Firtel, R. A. (1988). An 80-bp *cis*-acting regulatory region controls cAMP and development regulation of a prestalk gene in *Dictyostelium*. Genes Dev. *2*, 294–304.

Devreotes, P. N. (1994). G protein–linked signaling pathways control the developmental program of Dictyostelium. Neuron *12*, 235–241.

Dharmawardhane, S., Cubitt, A., and Firtel, R. (1994). Regulatory role of Gα1 subunit in controlling cellular morphogenesis in *Dictyostelium*. Development *120*, 3549–3561.

Dynes, J. L., Clark, A. M., Shaulsky, G., Kuspa, A., Loomis, W. F., and Firtel, R. A. (1994). LagC is required for cell–cell interactions that are essential for cell-type differentiation in *Dictyostelium*. Genes Dev. *8*, 948–958.

Fosnaugh, K. L., and Loomis, W. F. (1993). Enhancer regions responsible for temporal and cell-type-specific expression of a spore coat gene in *Dictyostelium*. Dev. Biol. *157*, 38–48.

Gomer, R., Datta, S., Mehdy, M., Crowley, T., Sivertson, A., Nellen, W., Reymond, C., Mann, S., and Firtel, R. A. (1985). Regulation of cell-type specific gene expression in *Dictyostelium*. Cold Spring Harbor Symp. Quant. Biol. *50*, 801–812.

Haberstroh, L., and Firtel, R. A. (1990). A spatial gradient of expression of a cAMP-regulated prespore cell-type-specific gene in *Dictyostelium*. Genes Dev. *4*, 596–612.

Hadwiger, J. A., and Firtel, R. A. (1992). Analysis of Gα4, a G-protein subunit required for multicellular development in *Dictyostelium*. Genes Dev. *6*, 38–49.

Hadwiger, J., Lee, S., and Firtel, R. (1994). The Gα subunit Gα4 couples to pterin receptors and identifies a signaling pathway that is essential for multicellular development in *Dictyostelium*. Proc. Natl. Acad. Sci. USA *91*, 10566–10570.

Haribabu, B., and Dottin, R. P. (1986). Pharmacological characterization of cyclic AMP receptors mediating gene regulation in *Dictyostelium discoideum*. Mol. Cell. Biol. *6*, 2402–2408.

Hjorth, A. L., Khanna, N. C., and Firtel, R. A. (1989). A *trans*-acting factor required for cAMP-induced gene expression in *Dictyostelium* is regulated developmentally and induced by cAMP. Genes Dev. *3*, 747–759.

Hjorth, A. L., Pears, C., Williams, J. G., and Firtel, R. A. (1990). A developmentally regulated *trans*-acting factor recognizes dissimilar G/C-rich elements controlling a class of cAMP-inducible *Dictyostelium* genes. Genes Dev. *4*, 419–432.

Insall, R. H., Soede, R. D. M., Schaap, P., and Devreotes, P. N. (1994). Two cAMP receptors activate common signaling pathways in *Dictyostelium*. Mol. Biol. Cell *5*, 703–711.

Jackson, C., Konopka, J., and Hartwell, L. (1991). S. cerevisiae α pheromone receptors activate a novel signal transduction pathway for mating partner discrimination. Cell *67*, 389–402.

Jermyn, K. A., Berks, M., Kay, R. R., and Williams, J. G. (1987). Two distinct classes of prestalk-enriched mRNA sequences in *Dictyostelium discoideum*. Development *100*, 745–755.

Johnson, R. L., van Haastert, P. J. M., Kimmel, A. R., Saxe, C. L., Jastorff, B., and Devreotes, P. N. (1992). The cyclic nucleotide specificity of three cAMP receptors in *Dictyostelium*. J. Biol. Chem. *267*, 4600–4607.

Johnson, R. L., Saxe, C. L., Gollop, R., Kimmel, A. R., and Devreotes, P. N. (1993). Identification and targeted gene disruption of cAR3, a cAMP receptor subtype expressed during multicellular stages of *Dictyostelium* development. Genes Dev. *7*, 273–282.

Kay, R. R. (1992). Cell differentiation and patterning in *Dictyostelium*. Curr. Opin. Cell Biol. *4*, 934–938.

Kesbeke, F., Snaar-Jagalska, B. E., and van Haastert, P. J. M. (1988). Signal transduction in *Dictyostelium fgd A* mutants with a defective interaction between surface cAMP receptors and a GTP-binding regulatory protein. J. Cell Biol. *107*, 521–528.

Kubohara, Y., and Okamoto, K. (1994). Cytoplasmic Ca^{2+} and H^+ concentrations determine cell fate in *Dictyostelium discoideum*. FASEB J. *11*, 869–874.

Kumagai, A., Mann, S. K., Pupillo, M., Pitt, G., Devreotes, P. N., and Firtel, R. A. (1988). A molecular analysis of G proteins and control of early gene expression by the cell-surface cAMP receptor in *Dictyostelium*. Cold Spring Harbor Symp. Quant. Biol. *2*, 675–685.

Kumagai, A., Pupillo, M., Gundersen, R., Miake-Lye, R., Devreotes, P. N., and Firtel, R. A. (1989). Regulation and function of Gα protein subunits in *Dictyostelium*. Cell *57*, 265–275.

Kumagai, A., Hadwiger, J. A., Pupillo, M., and Firtel, R. A. (1991). Molecular genetic analysis of two Gα protein subunits in *Dictyostelium*. J. Biol. Chem. *266*, 1220–1228.

Lilly, P. J., and Devreotes, P. N. (1994). Identification of CRAC, a cytosolic regulator required for guanine nucleotide stimulation of adenylyl cyclase in *Dictyostelium*. J. Biol. Chem. *269*, 14123–14129.

Lilly, P. J., Wu, L. J., Welker, D. L., and Devreotes, P. N. (1993). A G-protein β-subunit is essential for *Dictyostelium* development. Genes Dev. *7*, 986–995.

Mann, S. K., and Firtel, R. A. (1987). Cyclic AMP regulation of early gene expression in *Dictyostelium discoideum*: mediation via the cell surface cyclic AMP receptor. Mol. Cell. Biol. *7*, 458–469.

Mann, S. K., and Firtel, R. A. (1989). Two-phase regulatory pathway controls cAMP receptor-mediated expression of early genes in *Dictyostelium*. Proc. Natl. Acad. Sci. USA *86*, 1924–1928.

Mehdy, M. C., and Firtel, R. A. (1985). A secreted factor and cyclic AMP jointly regulate cell-type-specific gene expression in *Dictyostelium discoideum*. Mol. Cell. Biol. *5*, 705–713.

Mehdy, M. C., Ratner, D., and Firtel, R. A. (1983). Induction and modulation of cell-type specific gene expression in Dictyostelium. Cell *32*, 763–771.

Milne, J. L. S., Wu, L., Caterina, M. J., and Devreotes, P. N. (1995). Seven helix cAMP receptors stimulate Ca^{2+} entry in the absence of functional G proteins in *Dictyostelium*. J. Biol. Chem., in press.

Mukoyama, M., Nakajima, M., Horiuchi, M., Sasamura, H., Pratt, R., and Dzau, V. (1993). Expression cloning of type 2 angiotensin II receptor reveals a unique class of seven-transmembrane receptors. J. Biol. Chem. *268*, 24539–24542.

Oyama, M., and Blumberg, D. D. (1986). Interaction of cAMP with the cell-surface receptor induces cell-type specific mRNA accumulation in *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. USA *83*, 4819–4823.

Palczewski, K., Buczylko, J., Kaplan, M. W., Polans, A. S., and Crabb, J. W. (1991). Mechanism of rhodopsin kinase activation. J. Biol. Chem. *266*, 12949–12955.

Pears, C. J., and Williams, J. G. (1987). Identification of a DNA sequence element required for efficient expression of a developmentally regulated and cAMP-inducible gene of *Dictyostelium discoideum*. EMBO J. *6*, 195–200.

Pears, C. J., and Williams, J. G. (1988). Multiple copies of a G-rich element upstream of a cAMP-inducible *Dictyostelium* gene are necessary but not sufficient for efficient gene expression. Nucl. Acids Res. *16*, 8467–8486.

Powell-Coffman, J., Schnitzler, G., and Firtel, R. (1994). A GBF-binding site and a novel AT element define the minimal sequences sufficient to direct prespore-specific expression in *Dictyostelium*. Mol. Cell. Biol. *14*, 5840–5849.

Saran, S., Nakao, H., Tasaka, M., Iida, H., Tsuji, F. I., Nanjundiah, V., and Takeuchi, I. (1994). Intracellular free calcium level and its response to cAMP stimulation in developing *Dictyostelium* cells transformed with jellyfish apoaequorin cDNA. FEBS Lett. *337*, 43–47.

Schaap, P., and van Driel, R. (1985). Induction of post-aggregative differentiation in *Dictyostelium discoideum* by cAMP: evidence for the involvement of the cell surface cAMP receptor. Exp. Cell Res. *159*, 388–398.

Schaap, P., van Lookeren Campagne, M. M., van Driel, R., Spek, W., van Haastert, P. J. M., and Pinas, J. (1986). Postaggregative differentiation induction by cyclic AMP in *Dictyostelium*: intracellular transduction pathway and requirement for additional stimuli. Dev. Biol. *118*, 52–63.

Schlatterer, C., Knoll, G., and Malchow, D. (1992). Intracellular calcium during chemotaxis of *Dictyostelium discoideum*: a new Fura-2 derivative avoids sequestration of the indicator and allows long-term calcium measurements. Eur. J. Cell Biol. *58*, 172–181.

Schnitzler, G. R., Fischer, W. H., and Firtel, R. A. (1994). Cloning and characterization of the G-box binding factor, an essential component of the developmental switch between early and post-aggregative development in *Dictyostelium*. Genes Dev. *8*, 502–514.

Segall, J., Kuspa, A., Shaulsky, G., Ecke, M., Maeda, M., Gaskins, C., Firtel, R., and Loomis, W. (1995). A MAP kinase necessary for receptor-mediated activation of adenylyl cyclase in *Dictyostelium*. J. Cell Biol., in press.

Soede, R. D. M., Insall, R. H., Devreotes, P. N., and Schaap, P. (1994). Extracellular cAMP can restore development in *Dictyostelium* cells lacking one, but not two subtypes of early cAMP receptors (cARs): evidence for involvement of cAR1 in aggregative gene expression. Development *120*, 1997–2002.

Sun, T. J., and Devreotes, P. N. (1991). Gene targeting of the aggregation stage cAMP receptor cAR1 in *Dictyostelium*. Genes Dev. *5*, 572–582.

Town, C., and Gross, J. (1978). The role of cyclic nucleotides and cell agglomeration in postaggregative enzyme synthesis in *Dictyostelium discoideum*. Dev. Biol. *63*, 412–420.

Williams, J. G. (1991). Regulation of cellular differentiation during *Dictyostelium* morphogenesis. Curr. Opin. Genet. Dev. *1*, 358–362.

Williams, J. G., Ceccarelli, A., McRobbie, S., Mahbubani, H., Kay, R. R., Farly, A., Berks, M., and Jermyn, K. A. (1987). Direct induction of Dictyostelium prestalk gene expression by DIF provides evidence that DIF is a morphogen. Cell *49*, 185–192.

Wu, L., and Devreotes, P. N. (1991). *Dictyostelium* transiently expresses eight distinct G-protein α-subunits during its developmental program. Biochem. Biophys. Res. Commun. *179*, 1141–1147.

Wu, L., Gaskins, C., Gundersen, R., Hadwiger, J., Johnson, R., Pitt, G., Firtel, R., and Devreotes, P. (1994a). Signal transduction by G-proteins in *Dictyostelium discoideum*. In Handbook of Experimental Pharmacology, Volume 108: GTPases in Biology II, B. Dickey and L. Birnbaumer, eds. (Berlin: Springer-Verlag), pp. 335–349.

Wu, L., Gaskins, C., Zhou, K., Firtel, R., and Devreotes, P. (1994b). Cloning and targeted mutations of Gα7 and Gα8, two developmentally regulated G-protein α-subunit genes in *Dictyostelium*. Mol. Cell. Biol. *5*, 691–702.

Wu, L.-J., Kuwayama, H., van Haastert, P. J. M., and Devreotes, P. N. (1995). The G protein β-subunit is essential for multiple responses to chemoattractants in *Dictyostelium*. J. Cell Biol., in press.